(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) EP 0 822 403 A1

# (12) EUROPEAN PATENT APPLICATION

D 1

(43) Date of publication:
**04.02.1998 Bulletin 1998/06**

(51) Int. Cl.[6]: **G01N 1/30**

(21) Application number: **96112571.3**

(22) Date of filing: **02.08.1996**

(84) Designated Contracting States:
**AT BE CH DE DK ES FI FR GB GR IE IT LI LU MC NL PT SE**

(71) Applicant: **Milestone S.r.l.**
**24010 Sorisole (BG) (IT)**

(72) Inventors:
- **Lautenschläger, werner**
**D-88299 Leutkirch (DE)**
- **Visinoni, Francesco**
**Mozzo (Bergamo) (IT)**

(74) Representative:
**Schmidt-Evers, Jürgen, Dipl.-Ing. et al**
**Patentanwälte Mitscherlich & Partner,**
**Sonnenstrasse 33**
**80331 München (DE)**

## (54) Process for processing organic specimens

(57) This invention relates to the processing of organic tissues to prepare them for further investigation such as by light microscopy, electron microscopy, microwave staining, immunostaining, decalcification, hybridisation etc.

The present invention discloses a process for processing organic tissue, which comprises fixing an organic sample, dehydrating and clearing the sample and subsequently infiltrating and embedding the sample in a supporting medium to prepare it for further examination wherein the steps of dehydrating and clearing are carried out simultaneously.

EP 0 822 403 A1

## Description

The present invention relates to the processing of organic tissues to prepare them for further investigation. More specifically the present invention relates to a process for processing organic tissue via a combination of microwave, vacuum and pressure treatment comprising the steps of fixing an organic sample, dehydrating and clearing the sample and subsequently infiltrating and embedding the sample in a supporting medium to prepare it for basic Haematoxylin and Eosinstans and further techniques such as microwave staining, immunostaining, decalcification, hybridisation etc., for subsequent examination by light and electron microscopy.

Biopsies for diagnostic pathology and for anatomy pathology are presently prepared according to a conventional method, which is based on the following essential steps:

1. The tissue to be examined is treated with formaline or a saline solution to stop the process of decay and to stabilise the tissue so as to protect it against physical and chemical rigours of the processing.
2. In the second step the sample is dehydrated, i.e. some or all of the free water contained in the specimen is removed therefrom. During this procedure various cellular components are dissolved by the dehydrating fluids, such as lipids, which are extracted, and water soluble proteins, which are dissolved in aqueous alcohols.
3. The third step includes the treatment of the tissue with another solvent, the so-called clearing agent. Clearing is the transition step between dehydration and infiltration with the supporting medium. Since many dehydrants are immiscible with said subsequently applied supporting medium, a solvent is used which is miscible both with the dehydrant and the embedding medium to facilitate the transition between dehydration and infiltration steps.
4. The last step in the processing of organic specimens involves infiltrating the tissue cavities and cells by the supporting substance and finally embedding the tissue in the medium which will provide sufficient external support during the ensuing processing.

These procedures show, however, a variety of disadvantages, such as a long processing time of the specimens which may amount up to 50 hours per sample. Furthermore, a huge amount of the dehydrating agent and the clearing agent has to be employed. In a normal procedure the specimens are treated several times with the dehydrating agent, e.g. ethanol in rising concentrations (up to 100 % ethanol), and subsequently several times with the clearing agent in order to get rid of the dehydrating agent. Since large amounts of solvents have to be used, said solvents even being of high purity, the costs of such procedures are rather high.

On the other hand it proved to be difficult in practice to properly process organic tissue samples having a substantial amount of lipids contained therein (fatty tissue). In this case, the free water to be removed is retained in the tissue to a large extent making the use of excessive amounts of dehydrating agent necessary, which eventually results in an inferior quality of the specimens. This fact was considered to be due to fat cells embodied in the respective specimens preventing a free migration of water out of the tissue.

An alternative technique has been developed to reduce the time delay between receipt of the biopsy material in the laboratory and the availability of the tissue for further examination, and to rapidly obtain thin slides of the respective material. The biopsies are frozen in liquid nitrogen and then cut at lower temperature in a cryostat microtome. However, the specimens processed according to this method show a rather poor quality.

Hence there was a need in the art for an improved process for processing organic samples which obviates the above problems.

According to an aspect of the present invention there is provided a process for processing organic tissue which comprises the steps of

(i) fixing an organic sample to be processed,
(ii) dehydrating the sample with a dehydration agent,
(iii) clearing the sample with an essentially lipophilic agent, and
(iv) impregnating the sample,
wherein said steps (ii) and (iii) are carried out simultaneously by applying a mixture of a dehydration agent and a clearing agent simultaneously to the sample.

Other aspects and advantages of the present invention will be readily apparent to the skilled person when reading the present specification together with the claims taking into account the accompanying drawings.

The first step in the processing of organic tissue is the fixation of the respective specimen. In the process of the present invention there is no particular limitation as to the fixation method utilized. Hence strategies commonly employed to ensure fixation may be used. Methods suitable in the process of the present invention include microwave irradiation of biopsy specimens in normal saline, continuing fixation on the tissue processor with one or more changes of the routine fixative, preferably at elevated temperatures of 40 to 60°C, secondary fixation in formol sublimate on the tissue processor or in alcoholic fixative which will complete fixation while initiating dehydration, fixing in buffered phenol-formaldehyde ph 7.0 and pH 5.5 sequence at 40°C, etc.

In the second step according to the present process the specimens are treated with a combination of a dehy-

drating agent and a clearing agent.

As the dehydrating agent to be used in the present process there may be mentioned compounds which are essentially of hydrophilic nature. Examples for said compounds comprise lower alcohols, preferably alcohols containing 1 to 8 carbon atoms, lower ethers, preferably ethers containing 1 to 8 carbon atoms, ketones, preferably acetone, phenols, anilines, tetrahydrofuran, 2,2-dimethoxypropan, 2,2 diethoxypropan and creosote. Since any of these compounds reveal specific advantages for a given purpose or for a specific tissue specimen, the person skilled in the art will select the appropriate one for the respective task, or a mixture thereof.

Especially preferred dehydrating agents include alcohols, such as methanol, ethanol, propanol, isopropanol, n-butanol and t-butanol, or mixtures of these. Isopropanol or a mixture of isopropanol and ethanol is most preferred.

As the clearing agent to be used together with the dehydration agent in the present process there may be mentioned compounds, which are essentially of amphiphilic or lipophilic nature, which agents should preferably be miscible with the respective dehydrating agent utilized. Examples for said clearing agents include aliphatic hydrocarbons, which may be straight or branched, and which may be substituted with halides, such as chlorine, bromine or iodine. Also aromatic hydrocarbons may be used, which may be substituted by a moiety, comprising from 1 to 4 carbon atoms or which may be substituted by halides, such as chlorine, bromine or iodine. Also esters and terpenes have been found to be suitable clearing agents in the present process.

Specific examples include toluene, xylene, benzene, petroleum, paraffin, carbon disulphide, chloroform, carbon tetrachloride, trichlorethane, n-butylacetate, amylacetate, methyl benzoate, methyl salicylate, cedarwood oil, clove oil, inhibisol (inhibisol is the tradename of a cleaning fluid consisting of 1,1,1-trichlorethane with inhibitors designed to reduce its toxicity), limonene, terpineol and mixtures thereof. Preferred agents are aliphatic and aromatic hydrocarbons with said aliphatic hydrocarbon having from 6 to 12 carbon atoms, preferably 6 to 10, most preferably n-hexane, n-heptane and n-octane, being more preferred.

Based on his general knowledge and the teaching of the present invention the person skilled in the art will select the appropriate combination of the dehydration agent and the clearing agent for a given purpose. Thus, when treating e.g. fatty tissue a combination of a dehydration agent and a clearing agent will be chosen which allows a rapid extraction of lipids.

Preferred combinations of a dehydration agent and a clearing agent are alcohols combined with aliphatic and/or aromatic hydrocarbons. A combination of alcohols, having 1 to 4 carbon atoms, together with aliphatic and/or aromatic hydrocarbons is more preferred with the combination of isopropanol with one or more of petroleum, ether, n-hexane, n-heptane, n-octane, chloroform and/or acetone being most preferred. Instead of using isopropanol alone a combination of ethanol and isopropanol may be successfully employed. The amount of the dehydrating agent and the clearing agent in the solvent combination utilized may vary depending on the tissue to be treated and the properties inherent to the respective agents. A ratio of dehydrating agent : clearing agent of 20-80 : 80-20 % by volume are within the scope of the present invention. A preferred ratio is 50 : 50 % more preferred 60 : 40 %. In case of the preferred mixture of ethanol and isopropanol and n-heptane and/or n-octane all constituents are preferably included in equal amounts by volume. Unexpectedly it has been shown that the use of isopropanol in the dehydration/clearing agent results in the sample to be easily cut for later investigation.

The dehydration/clearing step is preferably carried out at a pressure of from atmospheric pressure to 10 bar, preferably at slightly elevated pressure, i.e. 1.1 to 5 bar, and up to 10 bar. Treatment under pressure allows for the sample to be treated at higher temperatures, namely at a temperature of from room temperature up to 90°C, preferably 70 to 90°C, more preferably 70 to 85°C, most preferred at a temperature of from 80 to 85°C without causing the combination of dehydration/clearing agent to evaporate and disrupting the tissue structure. The pressure may be effected by simply heating the sample in a closed container with the evaporating liquids building up a slight pressure. It is, however, preferred to build up the pressure by introducing a gas in the container, such as an inert gas, preferably nitrogen gas or carbon dioxide.

Surprisingly it has been found that the above conditions of pressure and temperature also result in a smoother defatting of samples without causing a denaturation of proteins and cell structures in the sample. Thus the morphology of the cell is fully maintained. Further this step is preferably carried out in a microwave device since it has been shown that heating of the sample may smoothly be effected by microwave treatment

Before carrying out the impregnation step the sample is dried, said process being primarily effected by means of moderate heat, i.e. heating the sample at a temperature from 30° to 60°C, preferably 40° to 55°C, more preferably 45° to 50°C. In order to promote the removal of residual water and solvent the drying process should be carried out under reduced pressure, preferably at a pressure in the range of from 300 to 500 mbar, mor preferably at 100 mbar. Methods currently employed also involve a quick rinse of the moist tissue cassettes with alcohol, preferably ethanol, no less than 80% concentration.

In the impregnation step (iv) of the present process the sample is infiltrated by and embedded in a medium the properties of which should generally approach those of the tissues to be examined with regard to density, elasticity, plasticity, viscosity and adhesion and

should be harmless to the embedded material. Generally the infiltration medium and the embedding medium are equal, but they may be different as well. Suitable materials to be used as infiltration and/or as embedding medium include compounds selected from the group consisting of agar, gelatine, polyvinylalcohol, polyetherglycols, polyethylene glycol monostearate, diethylene glycol distearate, ester wax, polyester wax, nitrocellulose, paraffin wax or a mixture thereof. Additives may be added to the waxes or blends of waxes, such additives being e.g. Piccolyte 115, which is a thermoplastic terpene resin added at the rate of 5-10 % to the infiltrating wax, plastic polymers, such as polyethylene wax or dimethylsuloxide (DMSO).

When applying the supporting media to the sample vacuum should preferably be applied to remove air entrapped in the tissue and to remove residual solvents used in the preceding combined dehydration and clearing step. Thus the impregnation step is preferably conducted under vacuum, starting with a moderate vacuum of from about 500 to 200 mbar, preferably 400 to 200 mbar, most preferably 300 to 200 mbar, in order to carefully degas the molten paraffin applied to the sample. In a next step the vacuum is then lowered, preferably to about 100 mbar or even 50 mbar.

Alternatively the impregnation step may also be carried out by alternately applying a moderate pressure, such as from 1,1 to 5 bar , preferably 1,1 to 3 bar for a time period of about 5 minutes or less, preferably 3 or 4 minutes, more preferred about 2 minutes, and a moderate vacuum for about the same time period. The reduced pressure to be applied ranges of from 100 to 800 mbar, preferably from 100 to 500 mbar, more preferred from 200 to 300 mbar.

According to the present process it became possible to process a variety of tissues including difficult tissues, such as tissues which are largely composed of keratin, dense collagen, closely packed smooth muscle fibres, colloid areas of haemorrhage, thrombi or yolk (hard dense tissues), or mummified tissues or yolk rich tissues and also fatty tissues. In addition it could be shown that the processing time for the specimens could be reduced enormously to about 30 minutes for processing needle biopsies and about 60 to 90 minutes for standard type tissue specimens.

Surprisingly it has been shown that the properties of the respective dehydrating agents and clearing agents are not altered when being combined, thus allowing a simple and efficient dehydration and clearing at the same time. In case of fatty tissues it has furthermore been shown that dehydration may easily be accomplished since the clearing agent also serves for extracting lipid substances, resulting in a smooth removal of the water without deterioration of the structure of the tissue to be treated.

In a preferred embodiment the present process is carried out in a microwave apparatus. Microwave treatment has been shown to accelerate the action of cross-linking fixatives and also allows the removal of the dehydrating agent and the clearing agent to proceed more smoothly.

According to another aspect of the present invention there is provided an apparatus for carrying out the present process which comprises

(i) a microwave heating device comprising a sealable dome arranged therein,
(ii) a container arranged in said dome for loading the samples,
(iii) a supply means for supplying reagents to the sample, and
(iv) a line for applying vacuum to said dome,
wherein the dome comprises a plate and a liftable confining element, wherein said liftable element encloses the container arranged in said dome, and wherein the line for applying vacuum to said dome extends through said confining element.

In a preferred embodiment the vacuum pump unit controls both the evaporation of the dome and the up and down movement of the upper portion thereof. The carousel, on which the plate of the dome is arranged, may rotate by 360°C and the temperature may be measured by infrared radiation through a window in the dome.

The dome may be manufactured of any material which does not adversely interfere with the microwave treatment of the samples. Polypropylene has been proven to be the preferred material. For heating the sample it has been shown that the use of plates, consisting of teflon having carbon incorporated therein (WEFLON), which convert microwave radiation into heat, assist in evenly heating the sample to be processed. Thus the dome itself may comprise WEFLON, or plates made of WEFLON may be arranged around the container.

In a preferred embodiment for carrying out the present process a WEFLON plate is put on top of the container, while one or more WEFLON plates are arranged below the container.

The container may comprise one ore more places for arranging forms therein, e.g. cassettes, into which the samples to be processed have been transferred.

The control of the solution and of the paraffin within the container as well as the vacuum in the dome may be accomplished by a computer via a specifically designed software.

Other means for executing said apparatus and preferred embodiments thereof are illustrated in the copending PCT application WO95/19560, the contents of which is fully incorporated herein by way of reference.

The gas evacuated from the dome is preferably passed through a charcoal filter. An automatic solvent detector may be included in the present apparatus to signal the exhaustion of the charcoal.

With reference to the Figures:

Fig. 1 is a schematic drawing of an apparatus to be used in the present invention during the loading-unloading of the sample into and from the container.

Fig.2 is a schematic drawing of an apparatus to be used in the present invention during operation.

Fig.3 is a schematic drawing of the container in the microwave unit showing an array of cassettes immersed in a solvent mixture (Lavis solution: containing a three part mixture of ethanol, isopropanol and n-heptane) in a ratio of 1:1:1 during the operation. The temperature is measured by means of an IR-sensor arranged outside the container.

Fig.4 is a schematic drawing of the container in the microwave unit showing an array of cassettes immersed in paraffin.

The present invention is now further illustrated by way of example:

Biopsies are cut to appropriate size and introduced in a non-microwave absorbing plastic cassette. The cassette is placed on a PFTE (polytetrafluorethylene) based rack and the rack is introduced in a glass container with a PTFE cover. A solution consisting of n-heptane, ethanol and isopropanol is filled into the container up to a level to cover the cassette. The PTFE based cover is placed on the container.

The dome is lifted pneumatically and the container is introduced in the microwave unit. A program is set at 900 mbar, so as to provide a modest vacuum in order to remove vapor, and the temperature is set to be in the range of from 45 to 85°C.

In the first step the solution is heated. The organic solvent mixture extracts lipids from fatty tissue releasing the cells, so that under the influence of the microwave the temperature of the water in the cells increases, thereby reducing its viscosity and resulting in a migration of the water towards the external periphery of the cell from where it moves into the solvent solution. This happens in a temperature range which is physiological and not damaging to the tissues.

The dome is lifted automatically at the end of the first step. The container is taken out and the solution is transferred to another container. The rack is reintroduced in the same glass container, this time without cover. The container is placed again in the microwave unit and a drying program is started under from normal atmospheric pressure down to 100 mbar setting.

At the end of the drying step the container is taken out. Molten paraffin is added as well as heating elements of WEFLON at the bottom of the container itself. The addition of these heating elements is required to avoid solidification of paraffin which does not absorb microwaves. A WEFLON cover is placed on the glass container which is again placed in the microwave unit. The program with a temperature of approximately 62-65 and vacuum started, finally reaching a setting of 100 mbar. During this step any residual solvent mixture is substituted by molten paraffin. At the end of the third step the dome lifts automatically and the tissues within the cassettes are ready for the conventional embedding, cutting and staining procedures to obtain slides.

## Claims

1. A process for processing tissue comprising the steps of

   (i) fixing an organic sample to be processed,
   (ii) dehydrating the sample with a dehydration agent,
   (iii) clearing the sample with an essentially lipophilic agent, and
   (iv) impregnating the sample,
   wherein said steps (ii) and (iii) is carried out simultaneously by applying a mixture of a dehydration agent and a clearing agent simultaneously to the sample.

2. The process according to Claim 1, wherein said sample comprises non-fatty and/or fatty organic specimens.

3. The process according to any of the preceding claims, wherein the dehydration agent is selected from the group consisting of $C_{1-8}$-alcohols, $C_{1-8}$-ethers, ketones and phenols and anilines, tetrahydrofuran, 2,2-dimethoxypropan and 2,2-diethoxypropan and creosote or a mixture thereof.

4. The process according to claim 3, wherein the alcohol is selected from the group consisting of methanol, ethanol, propanol, isopropanol, n-butanol and t-butanol or a mixture thereof.

5. The process according to claim 3, wherein the ether is selected from the group consisting of glycolethers, 2-ethoxyethanol, dioxane and polyethylenglycol or a mixture thereof.

6. The process according to claim 3, wherein the ketone is acetone.

7. The process according to any of the preceding claims, wherein the clearing agent is selected from the group consisting of aliphatic, aromatic and chlorinated hydrocarbons, esters and terpenes or a mixture thereof.

8. The process according to claim 7, wherein the clearing agent is selected from the group consisting of toluene, xylene, benzene, petroleum, paraffin, carbon disulfide, chloroform, carbon tetrachloride, trichlorethane, n-butylacetate, amylacetate, methyl benzoate, methyl salicylate, cedarwood oil, clove oil, inhibisol, limonene and terpineol or a mixture

thereof.

9. The process according to Claim 1 or 2, wherein the dehydration agent comprises isopropanol and the clearing agent comprises n-heptane and/or n-octane.

10. The process according to claim 9, wherein the dehydration agent comprises ethanol and isopropanol.

11. The process according to claim 10, wherein the ethanol, the isopropanol and the clearing agent are used in equal amounts.

12. The process according to any of the preceding claims, wherein the impregnation step (iv) comprises embedding the sample in a medium, selected from the group consisting of agar, gelatine, polyvinylalcohol, polyetherglycols, polyethylene glycol monostearate, diethylene glycol distearate, ester wax, polyester wax, nitrocellulose, paraffin wax or a mixture thereof.

13. The process according to any of the preceding claims, wherein step (ii) and (iii) are carried out under pressure, preferably at a pressure in the range of from 1,1 to 10 bar.

14. The process according to claim 13, wherein step (ii) and (iii) are carried out at a temperature in the range of from 70 to 90°C

15. The process according to any of the preceding claims, wherein before performing step (iv) the sample is dried at reduced pressure, preferably at a pressure in the range of from 300 to 500 mbar.

16. The process according to any of the preceding claims, wherein the process is carried out in a microwave device.

17. The process according to any of the preceding claims wherein the impregnation step is carried out under alternant pressure and vacuum conditions.

18. Use of an apparatus for carrying out the process according to any of the preceding claims comprising

(i) a microwave heating device comprising a sealable dome arranged therein,
(ii) a container arranged in said dome for loading the samples,
(iii) a supply means for supplying reagents to the sample, and
(iv) a line for applying vacuum to said dome,
wherein the dome comprises a plate and a liftable confining element, said liftable element enclosing the container arranged in said dome, and wherein the line for applying vacuum to said dome extends through said confining element.

19. An apparatus for carrying out the process according to any of the claims 1 to 17, comprising

(i) a microwave heating device comprising a sealable dome arranged therein,
(ii) a container arranged in said dome for loading the samples,
(iii) a supply means for supplying reagents to the sample, and
(iv) a line for applying vacuum to said dome,
wherein the dome comprises a plate and a liftable confining element, said liftable element enclosing the container arranged in said dome, and wherein the line for applying vacuum to said dome extends through said confining element, and wherein said dome is lifted by the action of a vacuum pump.

20. An apparatus according to claim 19, wherein the container in said dome comprises one ore more WEFLON plates.

Figure 1

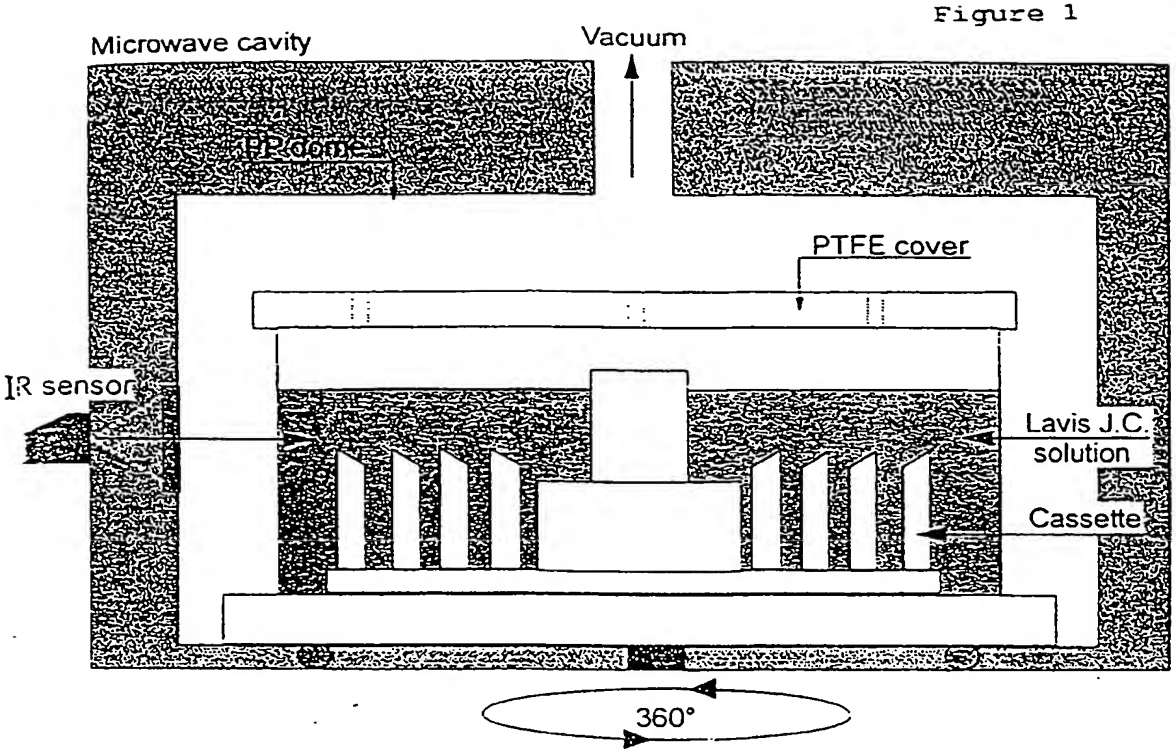



Figure 2

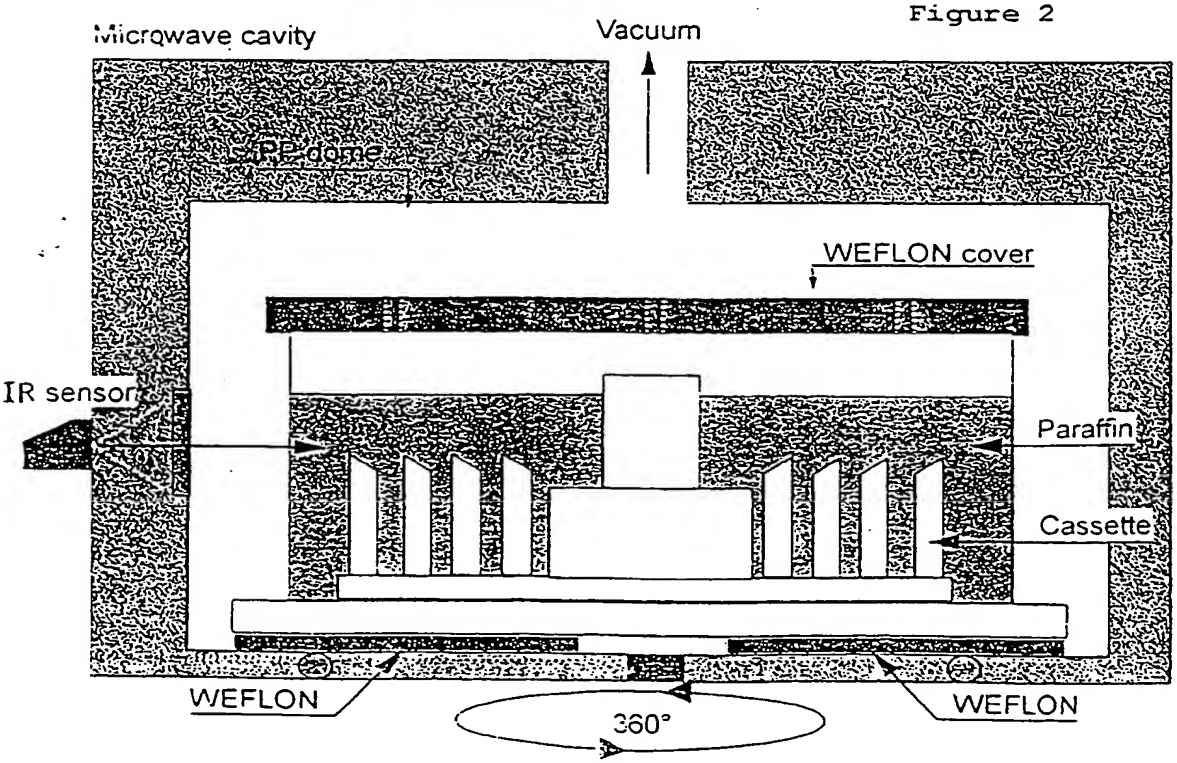
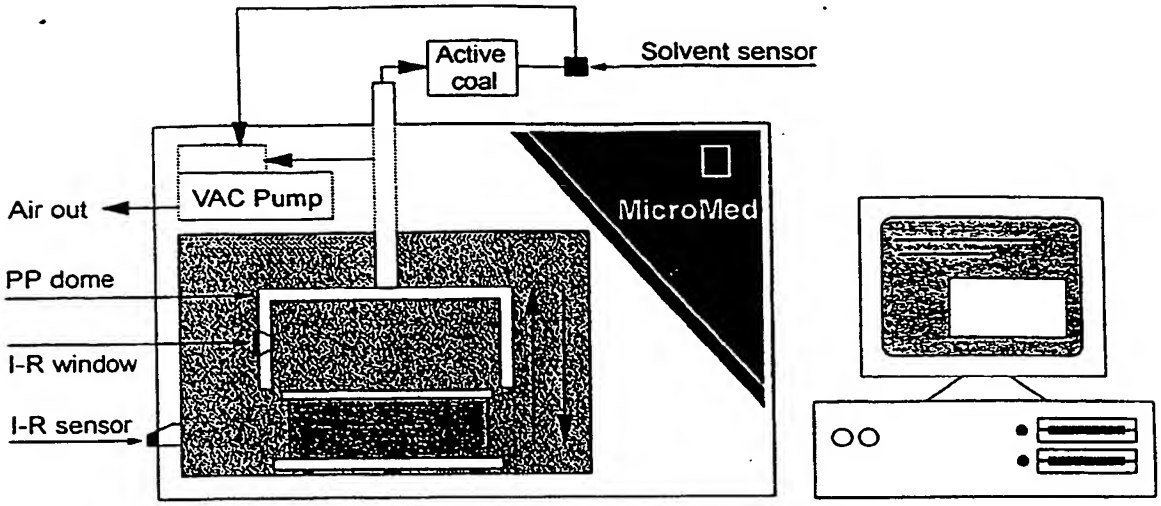
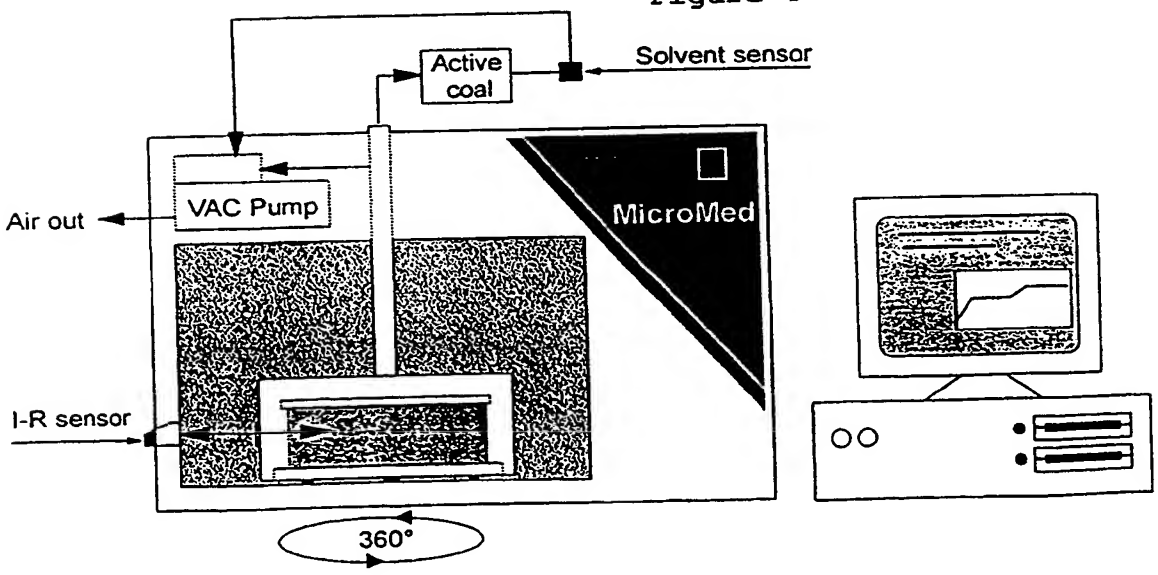


Figure 3




***Loading-unloading of container***

Figure 4




***In operating mode***

European Patent Office

# EUROPEAN SEARCH REPORT

Application Number
EP 96 11 2571

## DOCUMENTS CONSIDERED TO BE RELEVANT

| Category | Citation of document with indication, where appropriate, of relevant passages | Relevant to claim | CLASSIFICATION OF THE APPLICATION (Int.Cl.6) |
|---|---|---|---|
| Y | EUROPEAN JOURNAL OF MORPHOLOGY, vol. 33, no. 4, 1995, pages 349-358, XP000614291 M. E. BOON ET AL.: "The two-step vacuum-microwave method for histoprocessing." | 1-17 | G01N1/30 |
| X | * the whole document * | 18-20 | |
| Y | US-A-4 911 915 (FREDENBURGH JERRY L) 27 March 1990 * abstract * * column 4 * * column 7 * | 1-17 | |
| A | WO-A-96 18101 (CELLULAR DIMORPHISM INST) 13 June 1996 * page 6 * * page 7 * * page 15 * | 1-13 | |
| A | WO-A-86 06479 (RENVALL HENRIK GERHARD) 6 November 1986 * the whole document * | 17 | TECHNICAL FIELDS SEARCHED (Int.Cl.6) G01N |
| A | DE-A-38 24 936 (SCHUBERT WERNER) 22 March 1990 * the whole document * | 5 | |
| A | EP-A-0 350 271 (RAYTHEON CO) 10 January 1990 | | |
| D,A | WO-A-95 19560 (MLS GMBH ;LAUTENSCHLAEGER WERNER (DE); VISINONI FRANCESCO (IT); BO) 20 July 1995 * the whole document * | 18-20 | |

The present search report has been drawn up for all claims

| Place of search | Date of completion of the search | Examiner |
|---|---|---|
| THE HAGUE | 17 January 1997 | Cartagena y Abella,P |

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

EPO FORM 1503 03.82 (P04C01)